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Urinary excretion of C5b-9 reflects disease activity in passive Heymann nephritis

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Urinary excretion of C5b-9 reflects disease activity in passive Heymann nephritis. Passive Heymann nephritis (PHN) is a model of membranous nephropathy in rats in which glomerular injury is mediated by the terminal C5b-9 membrane attack complex of complement. This model has been shown to be associated with markedly elevated urinary excretion of C5b-9, compared to other experimental models of glomerulonephritis. To determine if urinary C5b-9 excretion could serve as an index of disease activity by correlating with the formation and quantity of glomerular subepithelial immune deposits in PHN, we measured urinary excretion of C5b-9 in PHN under several experimental conditions. In the heterologous phase a direct correlation was demonstrated between levels of urinary C5b-9 excretion and the amount of anti-Fx1A IgG deposited in glomeruli ($r = 0.85$). In the autologous phase, C5b-9 excretion correlated with the amount of deposit forming antibody present in the serum and resolved when antibody disappeared, despite persistence of glomerular deposits of antigen, antibody, C5b-9 and heavy proteinuria. Glomerular C3 deposits paralleled urinary C5b-9 excretion. Re-initiation of active deposit formation by a second injection of anti-Fx1A produced new C3 deposits and a marked rise in C5b-9 excretion. Finally, complete abrogation of deposit formation by transplanting PHN kidneys into normal recipients also halted C5b-9 excretion. Our findings demonstrate that urinary excretion of C5b-9 is a sensitive index of on-going immunologic disease activity in the PHN model of membranous nephropathy.

Membranous nephropathy (MN) is the commonest cause of idiopathic nephrotic syndrome in adults and is characterized by diffuse glomerular deposits of IgG and C3 in a subepithelial distribution [1]. The clinical course is quite variable with fluctuating amounts of proteinuria and an uncertain prognosis, both with respect to persistence of proteinuria and deterioration of renal function [2]. Multiple treatment protocols aimed at ameliorating the immune process which presumably underlies MN, usually employing steroids and/or cytotoxic agents [3, 4], have attempted to document beneficial effects of therapy using proteinuria and level of renal function as end points. However, since the putative pathogenic antibody in MN has not been identified, no data establishing any direct relationship between the immune disease mechanism and its consequences such as proteinuria and loss of renal function have been provided. In fact, studies of experimental MN [5, 6], as well as biopsy studies of patients with drug induced MN [7–9], suggest that proteinuria may persist long after activity of the disease process has subsided. Likewise, it is unclear whether the progressive deterioration of renal function and consequent sclerosis sometimes seen in MN result from a severe self-limited initial immune insult followed by secondary hemodynamic processes, or represent on-going immunologic disease activity. Thus, some index of activity of the underlying immune disease process would be extremely helpful in assessing the need for therapy with immunosuppressive agents as well as determining the response to such immunologic manipulation.

Passive Heymann nephritis (PHN) is a model of MN in rats which closely simulates idiopathic MN in man. Because of the striking clinical, histological and immunopathological similarities, most investigators believe that the disease mechanisms in PHN and MN are similar [reviewed in 10]. In PHN, subepithelial immune complex formation is initiated by binding of antibody to antigen(s) expressed on the membrane of glomerular epithelial cells [11, 12]. A consequence of in situ formation of immune complexes on the cell membrane is activation of complement with formation of C5b-9 complexes which mediate the increase in glomerular permeability observed [13, 14]. Recently, we reported that the presence of elevated urinary excretion of C5b-9 distinguishes PHN from other forms of experimental glomerulonephritis not involving epithelial cell membrane antigens [15]. The studies reported here extend this observation to demonstrate that elevated urinary excretion of C5b-9 correlates directly with the presence and quantity of on-going glomerular antibody deposition and complement activation in the PHN model. Thus, increased urinary C5b-9 excretion serves as both a marker for MN induced by antibody to a glomerular epithelial cell antigen and also as an indicator of the presence of on-going glomerular immune deposit formation.

Methods

Experimental design

To establish directly the relationship between increased urinary excretion of C5b-9 and on-going glomerular antibody deposition in PHN, four separate studies were carried out:

1.) To determine if the amount of urinary C5b-9 excretion in the urine correlated with the amount of antibody deposited in glomeruli during the heterologous phase of PHN. To examine the relationship between subepithelial immune deposit formation and the urinary excretion of C5b-9 in PHN, we measured

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urinary C5b-9 excretion following incremental doses of ^{125}I-labelled sheep anti-Fx1A IgG. Groups containing three male Sprague-Dawley rats each (Tyler, Bellevue, Washington, USA), weighing 180 to 200 g, received an i.v. dose of trace-labelled sheep anti-Fx1A IgG for 1 mg, 2 mg, 4 mg, 8 mg, 15 mg or 20 mg. Previous experiments had demonstrated that 7 mg or greater produced proteinuria exceeding 50 mg/24 hours by day five. Rats were placed in metabolic cages for two consecutive days beginning approximately 24 hours after injection of anti-Fx1A, and the urine collected was assayed for C5b-9 and creatinine content. At the termination of the second urine collection on day three, the quantity of antibody deposited in glomeruli was measured as described below.

2.) To determine whether urinary C5b-9 excretion correlated with glomerular antibody deposition during the autologous phase of PHN. The effect on urinary C5b-9 excretion of rat anti-sheep IgG binding to sheep anti-Fx1A IgG previously deposited in glomeruli was assessed. Nine rats received i.v. injections of 20 mg of sheep anti-Fx1A IgG, while nine control rats received 20 mg of normal sheep IgG. Rats were placed in metabolic cages every three days, and serial 24-hour urine determination of C5b-9, protein, and creatinine were made. Because quantitative measurement of autologous glomerular antibody deposition is technically difficult [16, 17], we assessed disease activity in this study by monitoring glomerular deposits of antigen (sheep IgG), antibody (rat IgG), C3, and C5b-9 by IF in sequential biopsies obtained every third day as well as by measuring serum levels of rat antibody to sheep IgG by ELISA. Biopsies of three rats from each group were obtained at each sampling point, sera and urine were obtained on all rats.

3.) To determine if urinary C5b-9 excretion could be increased by re-initiating active glomerular antibody binding. To test the hypothesis that re-development of active glomerular immune deposit formation in animals with PHN that had ceased excreting C5b-9 would result in another increase in urinary C5b-9 excretion, and to relate urinary C5b-9 to the quantity of antibody deposited in glomeruli, rats with established PHN induced by injection of 20 mg of anti-Fx1A were studied three weeks later when urinary C5b-9 excretion had decreased to nearly undetectable levels. Three groups of rats were studied, including controls ($N = 6$) injected with 20 mg of normal sheep IgG that received a second dose of 20 mg of normal sheep IgG three weeks later, a second control group ($N = 6$) that initially received 20 mg of sheep anti-Fx1A followed by 20 mg of normal sheep IgG i.v. three weeks later, and an experimental group ($N = 6$) that received 20 mg of anti-Fx1A followed by a second i.v. injection of ^{125}I anti-Fx1A at three weeks. Before the second injection in each group, three serial 20-hour urine collections were obtained for measurement of C5b-9, protein and creatinine. Immediately following re-injection of anti-Fx1A, another three serial urine collections were obtained for measurement of these same parameters. Kidney biopsies were then obtained and glomerular antibody binding measured as outlined below.

4.) To determine if cessation of glomerular antibody deposition would abrogate urinary C5b-9 excretion. To test the hypothesis that the complete cessation of on-going glomerular antibody deposition would result in disappearance of C5b-9 excretion in the urine, PHN was induced in 250 to 275 g Lewis rats by the i.v. injection of 15 mg of sheep anti-Fx1A IgG on two consecutive days. Twenty-four hour urine collections for C5b-9 and creatinine measurements were obtained on days 3 to 4, 4 to 5 and 5 to 6. On day six after antibody injection, nephritic kidneys from PHN rats ($N = 3$) were transplanted into a bilaterally nephrectomized normal Lewis rats. In one control group, a PHN kidney was transplanted into a bilaterally nephrectomized recipient ($N = 3$) that had PHN induced at the same time as the disease was induced in the donor. A second control group of PHN rats ($N = 4$) underwent unilateral nephrectomy on day six to exclude any effect of uninephrectomy alone on C5b-9 excretion.

Experimental details

Induction of PHN. In Sprague-Dawley rats, PHN was induced by the i.v. injection of a single dose of the IgG fraction of sheep anti-Fx1A prepared as described in detail elsewhere [6]. In Lewis rats the antibody was injected i.v. on two consecutive days. For glomerular antibody binding studies, anti-Fx1A IgG was trace-labelled with ^{125}I using the chloramine I method as modified by McConahey and Dixon [18].

Measurement of urinary C5b-9 excretion. All urine specimens were collected in 10% (vol/vol) buffer containing a mixture of protease inhibitors including 10 mM benzamidine (Sigma Chemical Co., St. Louis, Missouri, USA), 10 mM epsilon-aminocaproic acid (Sigma), 20 mM EDTA and 100 kallikrein inhibitor U/ml of aprotinin (Sigma). Detection of C5b-9 was performed by an enzyme linked immunoabsorbent assay (ELISA) that utilized two mouse monoclonal antibodies to a neoantigen of C5b-9 (2A1), and to C6 (3G11) which were raised, purified and characterized as described elsewhere [15]. Briefly, monoclonal antibodies were identified by their reactivity with rat EDTA-plasma and zymosan-activated rat serum separated by SDS-PAGE. Antibodies to human complement components which demonstrated cross reactivity with rat proteins were used as references [15]. 3G11 identified the same protein in EDTA-plasma as did the antibody to human C6, as well as a second high molecular weight band in zymosan activated serum, presumably representing the C5b-9 complex of complement. 2A1 reacted intensely with this same high molecular weight component in zymosan activated serum, but failed to react with EDTA-plasma and therefore presumably represents antibody to a C5b-9 neoantigen [15].

Microtitration plates (NUNC, Roskilda, Denmark) were coated with 4 μg/well 2A1 in carbonate buffer. After blocking with 0.05% casein (Sigma) in PBS serial dilutions of urine samples were incubated in wells overnight at 4°C. After washing with phosphate buffered saline, pH 7.2 (PBS)-0.05% Tween 20 (Sigma), biotinylated 3G11, 400 ng/well, was added for two hours, followed after washing by horseradish peroxidase coupled to strepavidin (Amersham, Arlington Heights, Illinois, USA). After one hour of incubation, the wells were again washed, followed by addition of 100 μl of solutions containing 0.2 mM 2.2'-azine-di-(3-athyl-benythia-zolinesulfonate) (ABTS, Boehringer Mannheim, Indianapolis, Indiana, USA) and 3.0 mM H_2O_2 in 100 mM acetate buffer at pH 5.0. The extinction at 405 nm was read after 30 minutes at room temperature using Dynatech MR560 microtiterplate reader (Dynatech). C5b-9 units were calculated using a previously defined, zymosan-activated rat serum reference standard [15].

Measurement of rat antibody to normal sheep IgG (NSIGG). All plasma samples were obtained through tail vein bleeds in 10% (vol/vol) 0.4 M EDTA. Antibody levels were measured by an ELISA utilizing NSIGG as both a coating and detecting

antigen. Microtitration plates (NUNC) were coated with 200 ng/well NSIGG in carbonate buffer. After blocking with 0.05% casein (Sigma) in PBS, serial dilutions of plasma samples were incubated in wells overnight at 4°C. After washing with PBS-0.05% Tween 20 (Sigma), 1.5 μg/well NSIGG biotinylated as previously described [15] was added for two hours, followed after washing by horseradish peroxidase coupled to avidin D (Vector, Burlingame, California, USA). After one hour of incubation, the wells were again washed, followed by addition of 100 μl of solution containing 0.2 mM ABTS and 3.0 mM H_2O_2 in 100 mM acetate buffer at pH 5.0. The extinction at 405 mm was read at 10 minutes using a Dynatech MR560 microtiterplate reader. Rat IgG antibody to sheep IgG served as a standard.

Glomerular antibody binding. Determination of glomerular antibody binding was performed as previously described [6]. Rats anesthetized with chloral hydrate had kidneys perfused with 60 mls of PBS in a retrograde fashion through the cannulated superior mesenteric artery to displace any retained blood prior to sacrifice. Cortical tissue was minced and passed through a no. 140 and no. 80 brass sieve. Glomeruli trapped on a no. 200 sieve were suspended in 5 ml of PBS-Tween. Identification and quantitation was based on light microscopic evaluation of five 25 μl samples placed on a Fuchs Rosenthal hemocytometer, with a total volume evaluated of 6.4 μl per sample. Gamma emission was then quantitated on the pelleted samples. Antibody bound to glomeruli was quantitated based on the specific activity of the injected antibody, the ratio of labeled to unlabeled antibody, and the gamma emission per isolated glomeruli.

Renal transplantation. All transplants were carried out in syngeneic Lewis rats. The left kidney was harvested using aseptic technique following induction of anesthesia with chloral hydrate. A cuff of vena cava and a segment of aorta were included to facilitate the vascular anastomoses. A generous length of ureter was obtained for direct implantation into the recipient bladder. The kidney was flushed in situ with 4°C normal saline containing lidocaine, 0.1 mg/ml and heparin, 10 U/ml. Topical papaverine was applied to the renal artery to prevent spasm. The kidney was stored on ice until the recipient was prepared. Preparation consisted of bilateral native nephrectomy, and a length of vena cava and aorta inferior to the native renal vessels were dissected free. End-to-side vascular anastomoses were performed using 10-0 monofilament nylon using the micro-surgical technique of Silber and Crudop [19] followed by ureterocystostomy. Recipients that exhibited gross hematuria or became oliguric (< 2 cc/24 hr) or anuric during the study period beginning 24 hours after transplant were excluded from the study.

Immunofluorescent (IF) studies. All renal biopsies of native kidneys were done under ether anesthesia, via a lateral flank incision. Biopsies obtained after transplantation were done under chloral hydrate anesthesia via a midline abdominal incision. Hemostasis was achieved with gelfoam (Upjohn, Kalamazoo, Michigan, USA). The tissue was flash frozen in dry ice-isopentane, and stored at −70°C until processed. Cryostat sections were cut, stained and studied with the fluorescein-conjugated IgG fractions of monospecific antisera to sheep IgG, rat IgG, and rat C3 (Cappel Laboratories, Inc., Cochraneville, Pennsylvania, USA) as described elsewhere [5]. Indirect IF for rat C5b-9 was performed by incubating tissue with an appropriate dilution of biotinylated 2A1 for 60 minutes followed by staining with fluorescein conjugated strepavidin (Amersham). The intensity of fluorescence was evaluated semiquantitatively as follows: 0, indistinguishable from control; trace, barely detectable; 1+, present but faint; 2+, definite; 3+, strongly positive; 4+, maximally positive.

Urine collections. All urine samples were obtained by placing rats in metabolic cages with free access to water but not food. Where consecutive daily urines were collected, rats were removed from the cage for four hours with free access to food and water daily prior to initiating the next collection period.

Miscellaneous measurements. Urine creatinine measurements were made using the picric acid method as described in detail elsewhere [20]. Urine protein excretion was measured by a sulfosalicylic acid method [21] using a whole serum standard (Lab. Trol., Dade Diagnostics, Aquado, Puerto Rico, USA).

Statistical analysis

Correlation of glomerular antibody binding with urinary C5b-9 excretion was performed using exponential regression [22]. All results of urine C5b-9/creatinine determinations and 24-hour urinary protein excretions are expressed as mean ± standard error. Differences between groups were analyzed using the Student's *t*-test where parametric relationships existed. Non-parametric data were analyzed using the Mann-Whitney test. P values < 0.05 are regarded as significant.

Results

The quantity of glomerular antibody deposited correlates directly with the amount of urinary C5b-9 excretion during the heterologous phase of PHN

To establish the relationship between the quantity of anti-Fx1A IgG bound in glomeruli during the heterologous phase of PHN and the amount of C5b-9 excreted in the urine, doses of ^{125}I anti-Fx1A IgG were administered which resulted in a range of antibody bound 72 hours later from 1.5 to 17.1 μg/76,000 glomeruli at 72 hours. Immunofluorescence studies performed on day 3 after injection on animals given equivalent doses of cold antibody demonstrated glomerular staining for sheep IgG and rat C3 and C5b-9 with only trace staining for rat IgG. No tubular brush border staining for sheep IgG or rat C5b-9 was detected. Measurements of urinary C5b-9 excretion from 24 to 72 hours demonstrated a strong direct correlation between the amount of ^{125}I IgG deposited in glomeruli and the two-day total urinary excretion of C5b-9 corrected for creatinine excretion (Fig. 1). All animals that received radiolabeled antibody had measurable deposits of antibody in the kidney. Animals that received doses too small to induce proteinuria (< 7 mg) all excreted C5b-9. Thus, urinary C5b-9 excretion correlated well with glomerular antibody binding even in the absence of detectable increases in urinary protein excretion.

Urinary C5b-9 excretion correlates with anti-IgG deposition during the autologous phase of PHN

The relationship between IF deposits of sheep IgG, rat IgG, C3 and C5b-9 as well as circulating levels of rat anti-sheep IgG, urinary protein excretion and urinary C5b-9 excretion in the autologous phase of PHN are presented in Figure 2. Staining for sheep IgG was maximal by day three and persisted unchanged for three weeks (Fig. 2A). Rat IgG staining was maximal by day nine and also persisted (Fig. 2A) as did glomerular deposits of

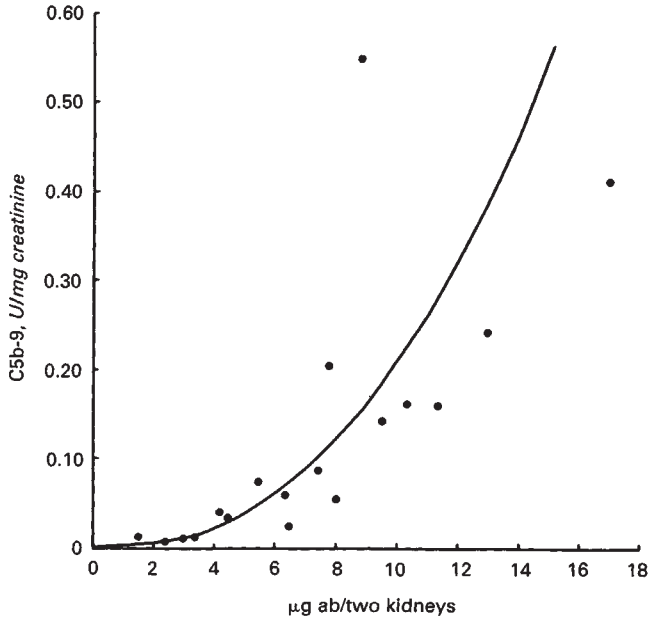

Fig. 1. *Relationship between glomerular antibody binding of anti-Fx1A (72 hours) and urinary excretion of C5b-9 in 18 rats.* The quantity of anti-Fx1A antibody bound correlated directly on an exponential scale with urinary excretion of C5b-9 as described by the equation $y = 0.906\ e^{.275\ x}$; $r = 0.88$; $P < 0.0005$. Each point represents data from one rat.

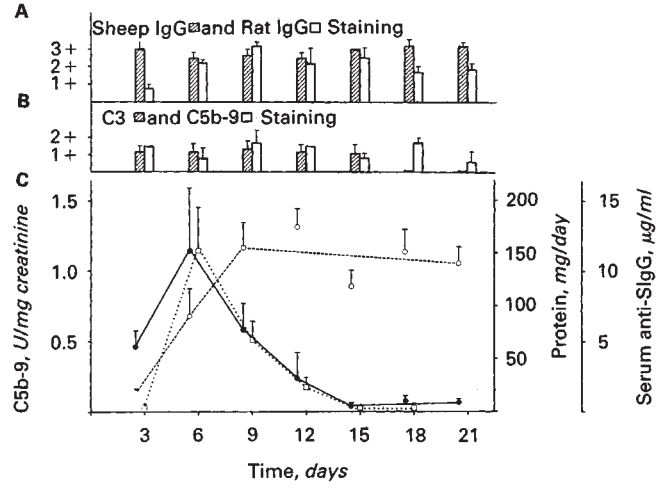

Fig. 2. *Time course of glomerular deposits of sheep IgG and rat IgG (A), rat C3 and C5b-9 (B) and rat anti-sheep IgG levels, urinary protein excretion, and urinary C5b-9 excretion during the autologous phase of PHN.* Urinary C5b-9 excretion parallels rat anti-sheep IgG levels and glomerular C3 deposits, while deposits of rat and sheep IgG, rat C5b-9 and proteinuria persist. Symbols in C are: (●) urinary C5b-9; (○) urinary protein; (□) serum anti-SIgG concentration.

C5b-9 (Fig. 2B). Proteinuria was present at three days, increased on day six, was maximal at day nine and persisted relatively unchanged through 21 days (Fig. 2C). In contrast, C5b-9 excretion peaked at day six and then diminished to normal levels by day 18 despite persistent proteinuria (Fig. 2C). Plasma antibody titers against sheep IgG given on day 0 were almost uniformly undetectable by day 3, were maximal on day 6 and then gradually decreased to undetectable levels again by

Table 1. Glomerular binding of anti-Fx1a antibody (Gab) in naive and autologous phase PHN kidneys 72 hours after injection

Dose	*N*	Kidney	Gab *μg antibody*	% Bound
15 mg	3	naive	8.660 ± 0.897	0.058 ± 0.006
20 mg	3	naive	13.769 ± 0.944	0.068 ± 0.015
20 mg	6	autologous PHN	201.500 ± 23.561	1.008 ± 0.118[a]

All values are mean ± SE. *N* = number of rats.
[a] $P < .01$

day 18, suggesting that urinary C5b-9 excretion reflected autologous phase disease activity induced by rat IgG antibody binding to planted sheep IgG antigen. Of interest, although deposits of antigen, antibody and C5b-9 persisted independently of apparent on-going immune deposit formation, glomerular deposits of C3 diminished (Fig. 2B) in parallel with urinary C5b-9 excretion (Fig. 2C).

Control rats injected with equivalent doses of normal sheep IgG, remained normal with respect to all parameters measured.

Urinary C5b-9 excretion correlates with the amount of antibody deposited in glomeruli after a second injection of anti-Fx1A

To further document the relationship between urinary C5b-9 excretion and active glomerular antibody deposition, rats in the autologous phase of PHN at three weeks in which urinary C5b-9 excretion had returned to normal values and glomerular C3 immunofluorescence had become negative were re-injected with anti-Fx1A. A prompt increase in urinary C5b-9 excretion occurred with redevelopment of glomerular C3 deposition (Fig. 3). As in the initial heterologous phase, the amount of C5b-9 excreted again correlated with the quantity of IgG bound in glomeruli 72 hours after injection. The quantity of anti-Fx1A antibody bound correlated directly on an exponential scale with urinary excretion of C5b-9 as described by the equation $y = 0.022\ e^{22.302\ x}$; $r = 0.82$; $P < 0.025$. As compared to glomerular binding of anti-Fx1A IgG in naive kidneys at 72 hours, which varied from 0.05 to 0.29% of the initially injected dose, the anti-Fx1A IgG bound to autologous phase PHN kidneys 72 hours after second injection was increased, ranging from 0.91 to 1.20% of injected dose (Table 1).

Urinary C5b-9 excretion ceases when glomerular antibody deposition stops

To further demonstrate that on-going glomerular immune deposit formation is required for increased urinary C5b-9 excretion, kidneys of rats with PHN were removed at day six when heterologous anti-Fx1A antibody deposition was ongoing, and urinary C5b-9 excretion was elevated, and were transplanted into nephrectomized normal recipients. With removal of kidneys from a nephritogenic environment, urinary C5b-9 excretion promptly ceased (Fig. 4). In contrast, kidneys placed in recipient rats that had earlier received the same injection of anti-Fx1A given to donors continued to excrete C5b-9 in the urine at levels identical to those in uninephrectomized donors (Fig. 4). In the normal recipients of PHN kidneys, C5b-9 excretion again increased five days after transplantation coincident with the appearance of glomerular deposits of rat

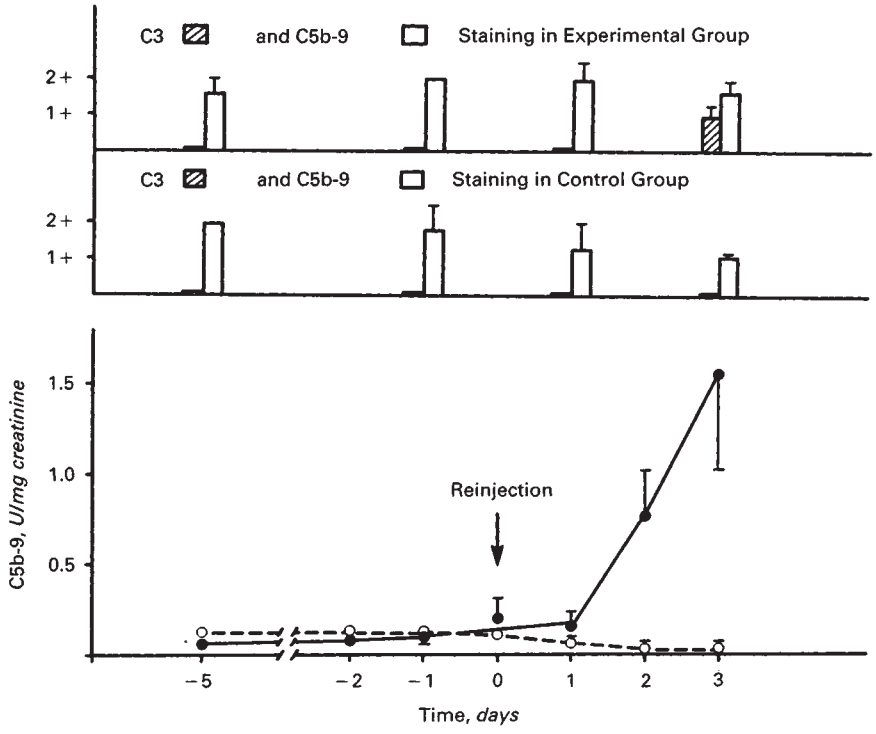

Fig. 3. *Effect of a second injection of anti-Fx1a and normal sheep IgG on: a. glomerular C3 deposition, and b. urinary excretion of C5b-9.*

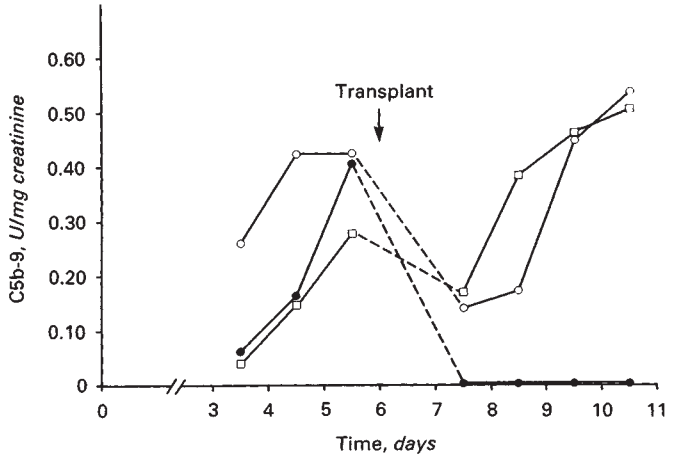

Fig. 4. *Effect of transplantation to a benign immunologic mileau,* N = *3 (●), transplantation to an identical immunologic mileau* N = *3 (○), and uninephrectomy* N = *4 (□) on urinary excretion of C5b-9 in PHN.*

IgG and therefore the onset of autologous phase antibody deposition (data not shown). Nephrectomized rats that received normal kidneys did not develop detectable urinary C5b-9 excretion (data not shown).

Discussion

Our studies confirm in several settings the suggestion from earlier work that urinary excretion of C5b-9 complexes reflects active immune deposit formation in glomeruli in a model of membranous nephropathy where these deposits result from antibody binding to a glomerular epithelial cell antigen [15]. When deposits are formed by this mechanism, the quantity of C5b-9 excreted was closely related to the quantity of antibody deposited regardless of whether that amount of antibody was sufficient to induce proteinuria. Moreover, C5b-9 excretion ceases as soon as antibody deposition is terminated, as evidenced both by termination of the autologous phase as well as the transplantation experiments. Previous studies have shown that the C5b-9 in the urine does not result from glomerular filtration of circulating C5b-9 complexes [15]. Moreover, the absence of detectable C5b-9 formation on proximal tubular brush borders, the onset of increased urinary C5b-9 excretion before proteinuria occurred, and the presence of increased C5b-9 excretion following deposition of quantities of antibody insufficient to cause proteinuria all suggest that the urinary C5b-9 complexes measured are of glomerular rather than tubular origin. However, the inducing antibody is reactive with tubular brush border, and some contribution of rapidly shed C5b-9 complexes formed on proximal tubular brush border cannot be excluded.

Of interest, our data also clearly demonstrate that urinary C5b-9 excretion coincides in time with the onset of antibody deposition during the autologous phase and correlates directly with the amount of antibody deposited, although the antibody in this phase is binding to exogenous antigen previously localized on the glomerular epithelial cell membrane or perhaps shed in a subepithelial distribution. Previous studies suggest that mediation of glomerular injury in this phase of disease is also C5b-9 dependent [14, 23, 24]. Moreover, increased C5b-9 excretion remains present in the autologous phase as long as antibody to sheep IgG is present in plasma and therefore presumably as long as deposit formation in glomeruli is on-going. The observation that urinary C5b-9 excretion in the autologous phase resolves by day 18, correlating in time with the disappearance of circulating antibody, suggests that deposit formation is self-limited despite the persistence of proteinuria. Clearly in this setting, urinary C5b-9 excretion served as a much more

sensitive indicator of disease activity than did glomerular deposits of antigen, antibody or proteinuria.

A related observation of some interest is that glomerular staining for C3 paralleled urinary C5b-9 excretion, suggesting that it too may represent an index of on-going disease activity. Thus C3 staining was present in the heterologous and early autologous phase of the disease, disappeared by day 18, and reappeared when passive administration of anti-Fx1A re-initiated disease with increased urinary C5b-9 excretion. C3 staining also disappeared within three days in the PHN kidneys transplanted into normal hosts. Observations of others in animals with glomerular C3 deposits suggest that C3 antigen is rapidly cleared when complement depletion is induced with cobra venom factor [25]. Thus the presence of C3 staining by IF suggests active on-going deposit formation and correlates with urinary C5b-9 excretion. Observations of C3 disappearance late in the course of autologous immune complex nephritis, therefore, suggest the cessation of the immunologic process late in that disease [26, 27]. Similarly in human membranous nephropathy, reports of wide variability in C3 presence and intensity [28–30] may likewise connote presence or absence of an on-going immunologic process.

An unrelated observation deserving comment relates to the marked differences in deposition of anti-Fx1A antibody in naive glomeruli versus autologous phase PHN glomeruli (Table 1). This 10- to 15-fold difference in binding could simply reflect the decreased barrier to filtration in the latter glomeruli, allowing more sheep IgG to penetrate the glomerular basement membrane and bind to the antigenic sites on the epithelial cell. An alternative hypothesis would be that the increase in antibody binding reflects up regulation of epithelial cell production of cell membrane antigen. Our data do not permit distinction between these possibilities.

The mechanism of urinary C5b-9 excretion in PHN has not been fully elucidated. Our previous studies suggest it occurs only when glomerular epithelial cell membrane insertion of C5b-9 occurs and that this phenomenon is prominent when subepithelial deposits form by antibody binding to a cell membrane antigen, but is absent or undetectable when deposits form by other mechanisms or at other sites [15]. Our current study also demonstrates that this phenomenon occurs when an antibody binds to a previously localized antibody on the surface of the epithelial cell. The studies of Kerjaschki and Farquhar and others suggest that the antigen is primarily GP330 [11, 12]. Recent immunoultrastructural studies demonstrate that the C5b-9 is inserted into the membrane of the glomerular epithelial cell in the region of the clathrin coated pits where deposit formation is initiated, and that it is endocytosed and transported in multi-vesicular bodies through the epithelial cell to be exocytosed into the urinary space [31]. This process appears to involve only C5b-9 and not antigen, antibody or C3 which are patched, capped and shed from the cell surface to form subepithelial immune complex deposits [32–34]. Thus the urinary excretion of C5b-9 appears to represent membrane insertion of the active complement complex whereas immune deposits not formed on the membrane surface appear to contain predominantly inactive S protein containing C5b-9 which cannot insert into cell membranes [31, 35]. An alternative explanation would be that the clathrin coated pit may contain vitronectin receptors for SC5b-9 that result in internalization and transcellular passage with subsequent exocytosis of the complexes [31].

The relationship between the epithelial cell processing of C5b-9 and the loss of glomerular barrier function leading to proteinuria and nephrotic syndrome is unclear. We and others have postulated that membrane inserted C5b-9 induces a sublytic effect on the cell leading to metabolic changes that ultimately result in loss of basement membrane functional integrity [14, 36]. Evidence supporting this concept includes the absence of apparent epithelial cell death in human and experimental membranous nephropathy as well as studies showing altered calcium influx [37, 38], increases in cAMP [37], stimulation of phospholipases [38], and altered type IV collagen production by glomerular epithelial cells exposed to C5b-9 in culture [39]. Glomerular mesangial cells exposed to sublytic concentrations of C5b-9 exhibit increased production of interleukin I [40] and reactive oxygen species [41]. However, the exact relationship of these cellular events to the alteration in glomerular permeability in this disease remains undefined.

The most important implication of our studies is the possibility that measurement of C5b-9 in human membranous nephropathy might produce information on the possible role of autoimmune mechanisms in the human disease as well as provide a non-invasive index of on-going disease activity. Experimental studies suggest that once the filtration barrier is lost in this disease, proteinuria may persist for extended periods of time in the absence of on-going deposit formation in the kidney [42]. Data from patients with drug-induced membranous nephropathy and membranous nephropathy in renal allografts suggest that the pathophysiology of the human lesion is similar [7–9, 43, 44]. While some controlled studies show benefit from steroid and immunosuppressive drug therapy in membranous nephropathy [3, 4], documentation of this benefit is complicated by the variable course of the disease, its indolent nature and the fact that no evidence of disease activity other than proteinuria and loss of renal function is available. It seems almost certain that significant numbers of patients are being treated with potentially toxic agents in the absence of any active on-going immune disease process. Our results provide a rationale for more definitive studies of the possibility that glomerular C3 deposits may also provide a helpful index of disease activity at the time of biopsy. If similar mechanisms are operative in human membranous nephropathy as recent data suggest [45], then both glomerular C3 staining, and urinary C5b-9 excretion could serve as indices of activity of the underlying immunologic disease.

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